Sepharose HR5/5 column and eluted by washing the column with 0.01 M potassium phosphate (pH 5.0) containing 0.1% CHAPS and ammonium sulfate at 20% saturation. Active fractions were immediately dialyzed against 0.01 M potassium phosphate (pH 5.0) containing 0.1% CHAPS. The purified enzyme gave a single protein band as judged by silver staining after SDS-PAGE [U. K. Laemmli, *Nature* **227**, 680 (1970)].

9. Aureusidin synthase activity was assayed by reversed-phase high-performance liquid chromatography as described [Method I in (*7*)]. One unit of enzyme was defined as the amount that catalyzed the formation of 1 nmol of aureusidin per minute. The specific activity of aureusidin synthase was expressed as units/mg of protein. The rate of oxygen consumption during aureusidin synthase reaction was also monitored with the Hansatech DW1/CB1D oxygen electrode system (Hansatech Instruments, Norfolk, UK) (*7*).
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21. PSORT analyses (http://psort.nibb.ac.jp/) did not predict the localization of aureusidin synthase in the plastids .

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Survival for Immunity: The Price of Immune System Activation for Bumblebee Workers

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Parasites do not always harm their hosts because the immune system keeps an infection at bay. Ironically, the cost of using immune defenses could itself reduce host fitness. This indirect cost of parasitism is often not visible because of compensatory resource intake. Here, workers of the bumblebee, *Bombus terrestris*, were challenged with lipopolysaccharides and micro–latex beads to induce their immune system under starvation (i.e., not allowing compensatory intake). Compared with controls, survival of induced workers was significantly reduced (by 50 to 70%).

Parasitic infections are pervasive, but hosts often show no obvious effects. Alas, this does not mean that parasites impose no fitness costs on their host, because the immune system is often able to keep the infection within bounds. Recent discussions in the field of evolutionary ecology have concentrated on the idea that the evolution of the immune system is traded off against other fitness components (*1*). In addition, the activation and use of the immune system are thought to be costly and therefore cannot be sustained simultaneously with other demanding activities (*2*). With such costs, the main effect of infection is not the direct damage by the parasite itself but the cost imposed when the host immune system is activated. Why, if these costs exist, are they not more often evident? One reason is that hosts may compensate for increased demand by increased resource intake. Costs are thus masked and no outward signs of a parasitic infection are observed, although the host pays a cost to prevent the establishment and spread of the parasite. To date, such fitness costs have only been shown indirectly, for example, by forcing the individual to increase its parental effort and measuring the corresponding decrease in the immune response (*2*).

Here, the survival cost for the activation of the immune system was analyzed when the host was denied compensation for increased demand. In particular, the host's condition was experimentally "frozen" by adopting a starvation protocol at the point when an "infection"—a standardized immunogenic challenge—occurred (*3*). When an individual is starved, any future allocation to defense reduces the resources available for other needs and thus eventually for maintenance and survival. The starvation paradigm also mimics some important ecological conditions, such as the natural occurrence of adverse weather and limited food availability, that are typical for most animal populations.

In this study, workers of the bumblebee, *Bombus terrestris* L., were used as hosts. Bumblebees are primitively eusocial insects inhabiting temperate habitats where weather conditions often vary over short time periods. Foraging activity is often interrupted by spells of rain and cold weather, leading to the starvation and demise of the colony if workers fail to collect sufficient amounts of pollen and nectar (*4*). Starved workers cannot survive for long (20 to 30 hours). In field populations, most workers are infected by some parasite but nevertheless show normal behaviors and activities (*5*). Bumblebee workers usually do not reproduce themselves. Hence, worker (inclusive) fitness is determined by their survival, and therefore any cost of immunity that reduces survival also reduces fitness (*6*). As in other insects, immunity in bumblebees is innate and based on both cellular (*7*) and humoral mechanisms (*8*). An immune response starts with the recognition of immunogens released by or present on the surface of parasites entering the host hemocoel. Various pathways of the immune system then become activated (*9*), leading to the destruction of the parasite and its removal by cellular reactions such as phagocytosis or encapsulation.

To measure the survival cost of the immune reaction, we experimentally activated the worker's immune system with two kinds of established immune elicitors. (i) Lipopolysaccharides (LPS; Sigma L-2755), i.e., surface molecules extracted from *Escherichia coli*. This nonpathogenic and nonliving elicitor is specifically recognized by pattern recognition proteins of the invertebrate immune system (*9*). LPS induces several pathways of the immune response (*8*, *10*) that persist over many hours (*11*). LPS is cleared from insect hemolymph by lipophorin, a transport protein that shuffles LPS to the fat body (*11*, *12*). Hence, the clearance of LPS should not involve processes that are responsible for clearing bacteria from the hemolymph such as phagocytosis. (ii) Sterile micro–latex beads (Polysciences; diameter 4.5 μm). These beads are a similar size to bacteria and are cleared from the hemolymph by a combination of processes (*8*, *10*), including phagocytosis. In both cases, the immune system is activated, but the artificial "parasite" is unable to generate any pathogenic effect.

A first experiment tested whether decreased survival might result from a toxic side effect of the immune elicitors, assuming that such effects would also decrease the survival of nonstarved animals. In addition, survival should then correlate with dose (*13*).

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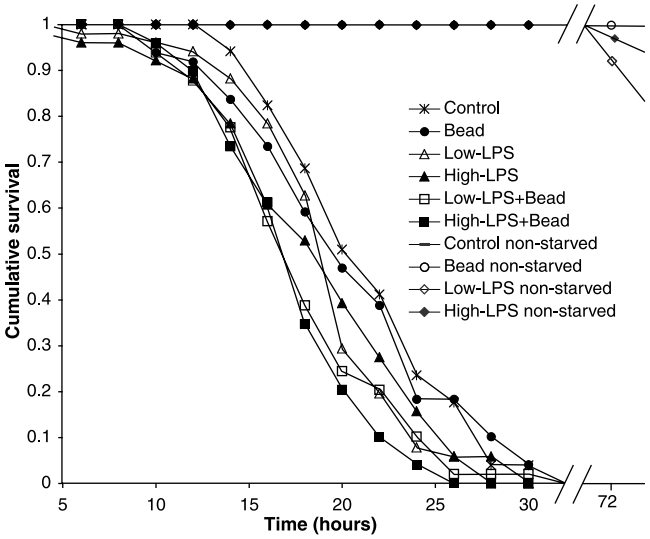

Fig. 1. Survival of workers under different treatments. The top, nearly horizontal lines refer to nonstarved animals (no difference found among treatments). Low-LPS + beads and high-LPS + beads refer to the combined injection of LPS and beads. Cox regression analysis for the starved animals (sloping lines) shows that injection of beads reduces survival by a factor of 1.56 (odds ratio; Wald statistic = 13.58, df = 1, $P < 0.001$). Similarly, injecting a low dose of LPS (odds ratio = 1.73; Wald = 13.18, df = 1, $P < 0.001$) or a high dose of LPS (odds ratio = 1.75; Wald = 13.86, df = 1, $P < 0.001$) reduces survival, but both the two doses produce a similar effect (comparing low-LPS with high-LPS: $P = 0.85$). Survival was also affected by colony of origin ($P < 0.001$) and its interaction with injection of beads ($P < 0.001$) and LPS ($P = 0.002$) and the corresponding three-way interaction (i.e., colony, beads, LPS: $P < 0.001$). All other terms were nonsignificant; in particular, there was no two-way interaction of beads and LPS [for further statistical details, see supplementary data available at *Science* Online (*18*)].

Survival of nonstarved workers for 72 hours after injection did not depend on dose of LPS [mean survival of workers in relation to dose: $r = -0.312$; $F(1,6) = 0.65$, $P = 0.45$, $N = 235$ workers] or on numbers of micro–latex beads [$r = 0.410$; $F(1,5) = 1.02$, $P = 0.36$, $N = 202$ workers] and, in particular, was not different from noninduced control animals (*13*). In addition, survival rate never dropped below 90%. Hence, neither LPS nor micro–latex beads appear to exert any toxic side effect, even when concentrations are 10-fold (LPS) or 100-fold (beads) as high as in the subsequent experiments.

Activation of the immune system by LPS and latex beads was measured by the antibacterial activity of hemolymph with a zone-inhibition assay (*14*). Antibacterial activity increased with dose of LPS [mean activity in relation to dose: $r = 0.895$; $F(1,6) = 24.25$, $P = 0.003$, $N = 216$ workers]. Induction, but no relation with dose [$r = 0.144$; $F(1,5) = 0.11$, $P = 0.76$, $N = 188$ workers], was found for latex beads. LPS and micro–latex beads may thus induce different pathways of the immune system.

The survival of challenged and control bees under starvation was tested in the same way. Two LPS treatments (low-LPS and high-LPS) and one bead treatment (beads) were used, in addition to a combined challenge of micro–latex beads and LPS (*15*). All inferences were made with Cox regression and with respect to the survival observed under the control (*16*). Three hundred workers were used in the analysis. As before, the activation of the immune system was checked by the zone-inhibition assay. The induction lasted as long as the experiment. Not surprisingly, starvation decreased survival time to a few hours (mean: 20.8 ± 0.67 hours, SE, $n = 51$, for control bees). However, survival time was reduced, by factors of 1.5 to 1.7 (odds ratios), for workers that were challenged by LPS or beads (Fig. 1). There was no significant difference in survival between high-LPS and low-LPS treatments. The addition of beads to LPS had an additive survival cost compared with LPS and beads alone (Fig. 1) (no significant two-way interaction terms in the Cox regression were found). Given the experimental paradigm and the biology of the species, the differences among colonies probably reflect genotypic differences among colonies.

Here, a hidden survival cost is demonstrated that is continuously paid to keep infections in check. It will go unnoticed when enough resources are available to compensate. Hence, even when no externally visible infections are observed in natural populations (*17*), such hidden costs can still have major effects on host survival strategies and ramify into many aspects of population biology and host-parasite coevolution. Future studies must therefore clarify to what extent resource allocation to defense is varied and whether such variation is in agreement with predictions of life history theory. Our results also suggest that the relative efficiencies of innate immune response in insects and acquired immunity in vertebrates (*2*) may be similar when costs are taken into account.

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13. Groups of 30 bumblebee workers were exposed to different doses of LPS (0, 0.1, 0.5, 1, 2, 3, 4, and 5 mg per milliliter) or latex beads (0, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 beads per milliliter), dissolved in Ringer, and injected as a total volume of 5 μl per insect. Controls only received Ringer but otherwise were treated identically. Before injection, bumblebee workers were anesthetized on ice and immobilized. The injections occurred into the hemocoel through the pleural membrane between the second and the third tergite, using a sterilized glass capillary that had been pulled out to a fine point. The inoculated bumblebees were kept under standard conditions in a climate chamber and fed with sugar water ad libitum.
14. For the assay, thoraces were separated from the rest of the body and homogenized in an Eppendorf tube containing 300 μl of Ringer solution. The homogenate was centrifuged (1300 g, 10 min, 4°C), and the supernatant were collected. The resulting solution was kept in the freezer (−80°C) for later tests. Test plates (diameter: 9 cm; Sterlin) were prepared by adding 0.05 ml of live *Arthrobacter globiformis* bacteria suspension (10^7 cells per milliliter) to 5 ml of sterile broth medium (10 g of bactotryptone, 5 g of yeast extract, 10 g of NaCl, 1000 ml of distilled water, pH 7.5), with 1% of bacto-agar at 45°C. Plates were swirled to disperse the bacteria and left to settle at room temperature. Ten test holes (diameter: 2 mm) per plate were made, and 2 μl of the test solution was added per hole. Plates were then incubated overnight at 28°C. During this time, the antibacterial substances in the hemolymph inhibited bacterial growth, leading to a circular, clear zone around each hole with a diameter proportional to the strength of the inhibition. The mean of the minimum and maximum diameters of each zone was used as data point.
15. For this experiment, random samples of 30 to 48 workers were removed from each of nine colonies kept in a climate chamber (constant 28°C, 60% relative humidity) and first fed ad libitum with sugar water and pollen. Each colony sample of workers was divided into six groups and assigned to one of the treatments, i.e., control, low-LPS (0.1 mg/ml), or high-LPS (0.5 mg/ml). Because no dose effect was observed for beads, only one concentration for beads was used (10^5 beads/ml); combinations were low-LPS + beads (0.1 mg/ml; 10^5 beads/ml) and high-LPS + beads (0.5 mg/ml; 10^5 beads/ml). After injection, workers were fed with sugar water for 1 hour before the starvation protocol; i.e., all feeding stopped after this period. For each individual, the time from starvation to death was recorded. Animals

that died during the initial 1-hour feeding period were discarded from the data set, on the assumption that this was due to non–treatment-related manipulation of the insects.
16. Cox regression allows the comparison of entire survival curves against an assigned reference survival function for different variables simultaneously. The best statistical model was found by a backward stepwise procedure. Treatment, colony (i.e., where the worker came from), and the interactions between these factors were entered into the full model. The treatments were coded as categorical variables (0, 1, 2) for LPS absent, low-LPS, and high-LPS, respectively, and with a second variable (0, 1) for beads being absent or present, respectively.
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Promiscuity and the Primate Immune System

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The behavioral and ecological factors involved in immune system evolution remain poorly explored. We present a phylogenetic analysis of white blood cell counts in primates to test three hypotheses related to disease risk: increases in risk are expected with group size or population density, exposure to soil-borne pathogens, and mating promiscuity. White blood cell counts were significantly greater in species where females have more mating partners, indicating that the risk of sexually transmitted disease is likely to be a major factor leading to systematic differences in the primate immune system.

Basal levels of white blood cells (WBC) are one of the first lines of defense against infectious disease (*1*). In mammals, disease risk is likely to vary with social, ecological, and sexual factors, providing predictions for differences in WBC counts across species. Social factors, such as group size and population density, are hypothesized to correlate with disease risk through increased transmission opportunities (*2*–*5*). Substrate use, an ecological factor, is another potential predictor of disease risk across species in that terrestrial species may be at greater risk of acquiring parasites through fecal contamination of the soil (*6*). Finally, greater frequency of sexual contact may lead to an increased risk of acquiring sexually transmitted disease (*7, 8*). Sexual contact frequency is quite variable across primates (*9, 10*). In gibbons (*Hylobates* spp.), for example, females are generally monogamous (*9*), whereas Barbary macaque (*Macaca sylvanus*) females mate with up to 10 males per day during estrus (*11*).

We used standard phylogenetic comparative methods to test whether evolutionary increases in the above-mentioned factors of disease risk are associated with evolutionary increases in WBC counts (*12*). We obtained mean WBC counts from adult females, mainly in zoos, with the use of the International Species Information System (*13*). These data are from healthy animals, with information available on 41 species representing all the major primate radiations. For each species, the mean number of samples was 112 (range, 11 to 357), and information was obtained from an average of 16 different institutions (range, 1 to 43). The advantage of using data from animals in captivity, as compared to those the wild, is that the health of individual animals is better ascertained, which is critical for estimating baseline WBC counts.

Higher WBC counts were found in species where females mate with more males (Fig. 1). However, bivariate regression analyses of independent contrasts found no support for group size, population density, percentage of time terrestrial, or body mass as predictors of overall WBC across primates (Table 1). In a multiple regression analysis with WBC as the dependent variable, only the number of mating partners was statistically significant (*14*).

We also tested the predictions using specific WBC types, including neutrophils, monocytes, and lymphocytes. Neutrophils and monocytes function in nonspecific phagocytosis, whereas lymphocytes are involved in adaptive immunity and in recognition of antigens. All of these WBC types contribute to protection against infectious disease (*1*) and are therefore predicted to increase with social, ecological, and sexual parameters. The effects of group size and population density remained unsupported in regression tests of particular WBC types. However, evolutionary increases in mating partner number were associated with increases in lymphocytes and monocytes, while a mean increase in neutrophils approached significance (Fig. 1). In addition, evolutionary increases in the percentage of time that primates spend on the ground were associated with significant increases in neutrophils [b = 0.069, $F(1,15) = 5.65$, $P = 0.02$]. It is well known that larger-bodied primates are more terrestrial (*15*). Further analysis revealed that body mass contributes significantly to neutrophil counts, but it was not possible to separate this effect from terrestriality (*16*).

We repeated analyses using a surrogate measure of female mating promiscuity that is quantitative rather than categorical and is based on estrous duration and testes mass. Longer estrous periods enable females to mate with multiple males (*10*). Testes mass, after correcting for body size, is a measure of the degree of sperm competition and therefore is a useful surrogate variable, in females, for the number of mating partners that a female is likely to have (*17*, *18*). To assess how these associated traits [$F(1,13) = 6.77$, $P = 0.02$] relate to disease risk, we used principal components analysis (PCA) of contrasts to capture in one variable the effects of estrous duration and residual testes mass. As our measure of mating propensity, we used the first principal component score, which explained 79.5% of the variation and had positive loadings for both variables; thus, a higher PCA score corresponds to increases in mating period and relative testes mass. The regression of WBC contrasts on first principal component scores was significantly positive (Fig. 2) [$F(1,13) = 20.8$, $P = 0.0003$]. In a multiple regression using this measure of mating promiscuity, group size, and percent time terrestrial on WBC contrasts, only promiscuity was statistically significant [b = 0.012, $F(1,7) = 5.80$, $P = 0.03$]. We also investigated patterns of male WBC counts and found similar patterns (*19*). In humans, WBC counts are more consistent with monogamy than promiscuity (*20*).

Our analyses demonstrate that basal immune system parameters vary among primates. The surprising result is that this variation appears to be driven by risk of acquiring sexually transmitted disease rather than disease that is transmitted as a function of social group size (*4*) or terrestrial locomotion (*6*). The precise reason for this result requires further study. It might be that sexually transmitted diseases are simply more common in nature than previously thought (*21*), or that behavioral mechanisms to avoid infectious disease (*22*, *23*) are less effective against sexually transmitted pathogens. Different components of the immune system may also be used to combat different types of disease (*1*). Thus, sexually transmitted diseases tend to be persistent and immuno-evasive (*21*), in the sense that they have mechanisms to avoid or combat induced responses. On the con-

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